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13. ABSTRACT *(Maximum 200 words)*

This report describes the work conducted in the second year of a two year IDEA for which a no cost extension has been approved. Our original proposal was funded to study a human estrogen regulated transcript, HEM45. Recent homology studies indicate that HEM45 is reated to a family of proteins that have an exonuclease domain. These include DNA proof-reading proteins and proteins associated with mitotic structures.

Studies in the reporting period have demonstrated altering patterns of HEM45 detectable in the cell cycle and an association with the mitotic spindle. These data strongly support a role for HEM45 in the cell cycle/proliferation. Ongoing studies address both the role and biochemical action of HEM45.

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B. TABLE OF CONTENTS:

C. INTRODUCTION
C.1 Basic characterization of HEM45 sequences
The focus of this study is HEM45. HEM45 was identified (1) as an estrogen regulated differential display product using a model system of estrogen receptor transfected HeLa cells (ER positive UP1 and matching ER negative, puromycin resistant CL lines).
The mRNA (GenBank U88964) encodes an ORF of 181 amino acid residues (~20,300 kDa). An Expressed Sequence Tag to rat HEM45 was found in the databases, facilitating use of the rat as an in-vivo model for a gene established as human-relevant by the initial cell experiments (1).
C.2 Homologues of HEM45
A similar human sequence (ISG20) was reported as interferon-induced in Daudi leukemic cells (2). It is likely the same sequence with minor sequencing conflicts. This form of HEM45 was reported as associated with lymphocyte-specific nuclear PML bodies. We dispute that and believe HEM45 is primarily cytoplasmic (see section D.1), though GFP fusions do localize to nucleoli see section D.2.
C.3 A family of related potential nucleases with diverse roles..... A context for HEM45 action
C.3.i Sequences comparisons: a relative of the 3'-5' proof reading exonuclease of DNA Polymerase I
We previously noted (1) that HEM45, at the protein level, had homology to several sequences, including Frt1, which is involved in a development switch for fruiting body formation in bracket fungus, and XPMC2 of Xenopus ovary which can rescue *S. pombe* from a 'mitotic catastrophe phenotype' (where the loss of ordering of replication and division leads to non-viability). The Xenopus ovary XPMC2 cDNA sequence (3) can rescue mutant *S. pombe* from a mitotic catastrophe phenotype that was created by disruption of cdc2 kinase regulation by eliminating Wee 1 and Mik 1 kinase activities. The cdc2 kinase is a key player in controlling cell-cycle transitions from both G1 to S phase and G2 to M (4)(5) in S. pombe (see section B.2), but the point of XPMC2 action is unknown. XPMC2 does not cause the inhibitory Tyr 15 phosphorylation on cdc2 kinase.

Our Biostatistics group, using Gibbs sampling approach (6), noted homology of HEM45 to the proofreading subunit of DNA polymerase δ and to ribonuclease T. This lead us to a report (7) that had used a different search approach (Hidden Markov Motifs) and had established a family of ~70 sequences that included HEM45 with the DNA polymerase 3'-5' exonucleases (see Fig. 1, apendices for excerpted alignment). Both alignment models clearly depended on conservation of features (Exo motifs I-III) previously noted as conserved in known proofreading subunits of DNA polymerase (8).

The family of ~70 sequences includes a variety of DNAses and RNAses, but did not include p53 which has a 3'-5' exonuclease proof reading function. The relationships are not strong, and not fully identified by systems such as blast or FastA. However, identification by two search algorithms, conservation of acidic residues known to be metal coordination sites in *E. coli* pol I and conservation of the 3 'exodomains' that include the active site features (notably a critical tyrosine or the substituting histidine depending on subfamily). General comments on structure are extrapolated from the solved crystal structure of *E. coli* DNA Pol I 3'-5' proof reading function.

The exonuclease motif appears in proteins of a variety of sizes, from 18,000 Da (OP18 [stathmin, oncoprotein 18] of leukemia cells) to 100 kDa (human polymyositis-Scleroderma autoantigen) to 175 kDa (*S. cerevisiae* SEP1). The 18 kDa OP18 and 20 kDa HEM45 are of the minimal size to contain the entire exonuclease motif. We note that the larger protein relatives of HEM45 may have properties derived from multiple domains, as recently reviewed (9) for the related Werner syndrome protein (where recessive alleles predispose for chromosomal instability and cancer) which includes helicase domains in addition to the exonuclease motif (and has a cell-cycle stage dependent association with nucleoli). It is also possible that the exonuclease motif has been recycled to novel functions during evolution; we do not envisage all members of this family as being proofreading subunits of DNA polymerase

C.3.ii Key points from family comparisons:
1.) The features that define the family are features found in Polymerase proof reading functions, making nuclease activity of HEM45 likely. The *S. cerevisiae* gene Pan2 (Fig. 1) was assigned to the same sequence-based subgroup as HEM45 and XPMC2 and is part of a poly A ribonuclease. Its inactivation causes a lengthening of cellular poly-A tails. It apparently encodes a 135 kDa protein with 3'-5' nuclease activity (10), this suggests that the subfamily can have nuclease activity, though the apparent promiscuous activities of many members of the family make it impossible to predict if this might be for DNA, RNA or hybrids. 2.) A feature of several members the family is a physical association with microtubule structures and mitotic control, as typified by OP18 of leukemia cells, Rec1 of the fungus *Ustilago maydis*, XPMC2 and Sep1 of *S. cerevisiae*. This was noted in the published report of the exonuclease family, without any knowledge of the association of HEM45 with mitotic microtubule structures (see sectionD.1). Some of the genes characterized as having a role as mitotic checkpoint proteins also have nuclease activities. These include REC1 and SEP1.

In summary, our experimental data (see D.1) indicate a linkage of HEM45 with mitotic structures by visualization techniques. HEM45 has closest homology to sequences having a role in control of cell division (XPMC2) and its sub-family that has at least one member with proven nuclease activity (PAN2).

C.3.iii Lower eucaryotic sequences with cell division role and nuclease activity
The biochemical actions of family members that were identified as having a role in mitosis (and/or meiosis) is confusing due to identification of activity on a variety of substrates. A recent paper thoroughly analyzed Rec1 of the fungus *Ustilago maydis* as a 3'-5' exonuclease on ordinary DNA templates, yet previous studies (11) showed a strong affinity to Z-DNA and this was thought significant for action in recombination. In addition the relative Sep1 of *S. cerevisiae* was identified as both a 5'-3' exonuclease and as 5'-3' exo ribonuclease (also active on ds-RNA and DNA-RNA hybrids (12)) and thought to have a role in recombination, and later identified as a DNase active on G4-tetraplex DNA (cutting at adjacent ss regions) by Wally Gilbert (13) - this alternate DNA structure being found associated with G-rich telomeric regions. Indeed, SepI was cloned at least 5 times for different reasons from yeast. Note that SEP1 of *S. pombe* is an unrelated, distinct, gene.

These sequences, and their role in cell division, were originally identified by genetic approaches. Mutation (truncation) of REC-1 caused UV sensitivity, including increased mutations rates. Cells with a mildly truncated (*rec1-1*) form of the 56 kDa *rec1* protein showed UV sensitivity with a 10-fold increase in mutation rates (14). A greater C-terminal deletion (*rec1-5*) had a 100-fold increase in mutation rates; this form lacked the exonuclease motif. The lines were equally sensitive to the killing effect of UV, suggesting that the repair (proofreading) role of *rec-1* was linked to the exonuclease domain, but that lethality effects were not. Enhanced sensitivity of cells with the mildly truncated *rec1-1* allele to effects of UV (death) could be mitigated by an inhibitor of microtubule formation which imposes an artificial block in G2 as chromosomes fail to separate. These also did not respond correctly to a Hydroxy-urea block of DNA synthesis, continuing cell division (15). This suggested a role of *rec-1* in sensing DNA damage and acting at a G2-mitotic checkpoint . This function of *rec-1* is lost on C-terminal deletion. Overall, data indicates a multi-functional role for *rec-1* in mitosis which partly involves the exonuclease domain. REC1 RNA expression was linked to G1/ S, as though in preparation for a role in mitosis. *Sep1* also appears to have an association with the mitotic microtubules, as mutants are hypersensitive to effects of benomyl, and show increased chromosome loss (16). However, Gilbert and coworkers attribute the latter to a role of *Sep1* in telomere shortening (13).

C.3.iv Stathmin/Oncoprotein 18: A mammalian relative with an important role in mitosis
OP18 is a cytoplasmic protein over-expressed in leukemic cells and leukemias. It is also expressed in developing neurons and a variety of tissues. It forms an association with α/β tubulin dimers, and perhaps other proteins, that affects their availability for incorporation into microtubule structure, with notable

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effects on mitotic spindle formation. Unphosphorylated OP18 promotes dissociation of tubulin dimers from microtubule structures. This activity is blocked by op18 phosphorylation. In growing tumor cells, phosphorylation is cell cycle dependent. Interference with OP18 levels or phosphorylation leads to a cell cycle block at G2/M and endoduplication (increasing nuclear DNA copy number due to lack of division) presumably reflecting OP18 influence on the dynamics of spindle microtubule polymerization. See (17) for a general review of OP18.

OP18 phosphorylation state is affected by both cell cycle dependent activities (including Cdc2 and cdk2-kinase (18)) and extra-cellular signals (including via MAP kinase pathways (19)). This may allow OP18 to function as an integrator of cellular cues. A range of kinases and phosphatase have been shown or are predicted to interact with OP18. These include KIS, with some homology to cdk2, which was identified in a two hybrid screen with OP18 as bait (20). We note that while nuclease activity of OP18 has not been tested/reported, KIS does have an RNA binding domain. Imaging techniques do not appear to have yet been used to characterize OP18/ stathmin localization in the cell or the cell cycle.

Based on the common, machine recognizable protein phosphorylation motifs (GCG motifs and prosite), HEM45 has equal or greater capacity than OP18 to be phosphorylated, but actual site utilization has not been tested for HEM45. That the regulation of OP18 is tied into cell cycle a forms a nice corollary to the observation that XPMC2 introduction to wee1/mik1 kinase defective *S. pombe* (where cdc2 regulation is disrupted) rescues from mitotic catastrophe.

C.4 HEM45 expression in cultured cells
C.4.i RNA In cultured cells
The HEM45 sequence is expressed in cells from a wide range of tissues and is generally estrogen regulated in ER +ve cells (1). Maximal induction is seen 6-10 h after a single treatment of E_2. The induction of HEM45 mRNA in UP1 HeLa is not blocked by cycloheximide indicating that induction in cultured cells is direct (transcriptional). Growth status/confluence did not affect RNA status, though we have not directly evaluated HEM45 mRNA expression in synchronized cells. RNA expression did not correlate with the aggressiveness of breast cultured lines (see first report for the IDEA), though that analysis did attempt to control the growth status of the cultures used to make RNA.

C.4.ii HEM45 protein expression in HeLa cells
Rabbit Polyclonal antisera were developed using HEM45 3-16 peptide (X826 antisera), peptide 100-116 and recombinant HEM45 (Y29 antiserum) as immunogen. Recombinant HEM45 was expressed in *E. coli* using the Studier T7 polymerase system pET15b vectors giving a His tag leader fusion. Antisera identified a ~20000 Da band in HeLa extracts.

Preliminary histochemistry of HEM45 showed strong staining in pre-confluent HeLa. Signal with HeLa at high density was much weaker. This was reported in last year's report.

In a separate experiment, plating of cells from highly dense cultures (to a situation where confluence is achieved in ~36 h) results with time in a strong elevation of protein expression, as measured in westerns. These points were covered in the year 1 report on this project. Apparently cell density affects HEM45 protein expression: either cell contact or reduced growth down-regulates HEM45 expression. This is in contrast our earlier (unpublished) data where cell density had *no* effect on HEM45 *mRNA* expression, and is confounding attempts to over express HEM45 in mammalian cells. We currently resolve this paradox by saying that estrogen induces HEM45 mRNA expression to ensure there is a sufficient HEM45 RNA to make the protein required by estrogen stimulated growth programs. We use an automobile analogy: transcription fills the gas tank with mRNA, but its the accelerator pedal that controls road speed, not the fullness of the gas tank.

C.5 HEM45 expression *in-vivo* in uterus

Although not part of the IDEA, data, from colleagues, on uterine expression of HEM45 in-vivo has influenced our concepts of HEM45 behaviour: in humans HEM45 antigen associates with the proliferative phase epithelium, and immediately adjacent underlying layers. In the rat peak expression is in the diestrous epithelium. Both of these localizations are in agreement with a role in proliferation. The epithelial layer in the rat diestrous phase was identified as the period/location of maximal mitoses (21), generally this period is under estrogen influence, with modulation of estrogen action by progestins.

D. BODY OF REPORT
Progress in the latest reporting period:
D.1 HEM45 Immunolocalization in the cell cycle of cultured tumor cells

HEM45 was evaluated in HeLa cells as part of tasks 7 and 13. Cytological examination of subcellular distribution of HEM45 showed that compartmentation of HEM45 changed during the cell cycle (Fig. 2). These data were collected on cells from preconfluent cultures synchronized with a double thymidine block. In *interphase*, HEM45 staining was found as fine speckles randomly, but exclusively, distributed (Fig. 2.a), through the cytoplasm. No intra-nuclear staining was seen. The punctate *interphase* pattern for HEM45 was distinct from that for anti-tubulin antibody staining of interphase microtubules (Fig. 3a&c).

As cells entered mitosis (in *prophase*, Fig. 2b), HEM45 staining became concentrated at the cytoplasmic side of the nuclear envelope. With breakdown of the nuclear envelope, HEM45 antibody staining was amorphous but surrounded *mitotic* spindle fibers, the element itself and spindle caps (Fig. 2c). As mitosis progressed, HEM45 moved towards the poles (anaphase, Fig. 2d). HEM45 staining was again found randomly distributed in the cytoplasm of telophase cells and in the midbody (not shown). The staining of HEM45 appeared to co-localize with spindle microtubules during *mitosis* (metaphase, Fig. 3b&d) but was more amorphous than that for tubulin. This suggests that HEM45 was associated with spindle structures but perhaps not directly associated with the central tubulin-based framework. At no stage of the cell cycle was HEM45 staining of chromosomes observed. On the contrary, chromosomes were visible by fluorescence microscopy due to their negative HEM45 staining. Disrupting the microtubules with nocodazole appeared to have little effect on the subcellular distribution in interphase cells, but caused randomization of HEM45 in mitotic cells (not shown). These data support the concept that HEM45 becomes microtubule-associated only during mitosis, and thus is not a typical microtubule-associated-protein.

Our subcellular localization is in conflict with the report on a probable HEM45 protein (pISG20) in Daudi cells (2). Mechti's lab reported that epitope tagged protein, from transiently transfected DNA ISG20 (HEM45) constructs, localized to lymphoid specific nuclear PML bodies. We did not find that HEM45 was nuclear by immunostaining in Daudi cells. The data from Mechti's lab may have been affected by either the of transient transfection plus an epitope tagged ORF (rather than probing the authentic protein). However, this paradox is still under analysis as we find nucleolar association of HEM45-GFP fusion (section D.2) in transiently transfected and stable cells. We suspect that they lost the cytoplasmic protein under their fixation method.

The biochemical basis for the association of HEM45 with spindle microtubules only during metaphase and anaphase is unknown but could be due to mitotic expression of a cellular component which binds HEM45 to spindle microtubules or to HEM45 post-translational modifications during mitosis, affecting its affinity for spindle components.

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D.2 HEM45-GFP cell lines and cell cycle expression

And our original aim 1 had the implicit assumptionsthat over-expression of HEM45 mRNA over-expression will result in protein over-expression and cause readily apparent effects on cell behavior. Although we have not made a comprehensive analysis of Tet regulated system effects on protein, we do find HEM45 protein expression is not strictly correlated to mRNA level. Eg previously reported (original application) lack of effect of cell density on HEM45 mRNA expression/ estrogen regulation contrasts to protein level effects of cell density (see year one report). In addition induction of growing cells carrying the regulated HEM45 construct did not change the apportionment of cells to cell-cycle stages (data not shown).

As an alternative model we have developed fusions of HEM45 ORF N-terminal to a Green fluorescent protein (GFP) cassette (HEM45-GFP fusion). This has application to both expression studies and to localization in living cells (following one cell though the whole cell cycle). The data should lead to better understanding of HEM45 regulation/action, and suggest optimal targets for testing effects of over-expression in the Tet-regulated system.

There are two assumptions/hypotheses in the use of GFP constructs:

1. That HEM45 regulation is 'independent' of transcription/ mRNA level- if this is the case, we should see that HEM45-GFP expression patterns are similar to those of HEM45 antigen (see above), this will indicate that the principal regulatory factors for HEM45 protein regulation lie beyond transcription (using the auto analogy, control is through the accelerator, providing there is gas (mRNA) in the tank, so we shouldn't notice the new gas tank, and a switch in gas brands). This is the alternative hypothesis to that in the Tet-regulated studies where we were seeking to perturb HEM45 expression.

2. HEM-GFP trafficking, turnover reflects the behavior of HEM45 rather than GFP. After creating the HEM45 lines we realised this was an important point for both expression and trafficking. In other work with Dr DePasquale (see below) we made a membrane (N-Cadherin) targeted fusion protein - in that case it was clear that the fusion was targeting the correct site (membrane), However both native GFP and HEM45 are cytoplasmic, making interpretation more problematic. We are currently making matching stable GFP lines to go with the HEM45-GFP fusion construct - so as to be able to at least partly cover both these points. However we do know that HEM45-GFP appears to have altered affinity for cell structures cf GFP; the HEM45 fusion is not lost on ethanol fixation, and it can be detected in nucleoli of some cells unlike GFP alone.

   One HEM45-GFP construct, with HEM45 N-terminal to GFP, has been created placing the HEM45 ORF N teriminal to GFP sequences in pEGF-N1, creating a construct expressing a fusion protein. The construct was introduced to HeLa cells in transient transfection assays, and stable puromycin lines created lines. Expression was evaluated by a colleague, Dr DePasquale, using fluorescence microscopy (Fig. 4) and also by flow cytometry (Fig. 5). Both techniques detect cells that are very bright and cells that are less bright (Fig. 4) , both populations fluoresce with greater intensity than untransfected Hela (see Fig. 5), and with a distinctly different pattern to autofluorescence (not shown). Some cells transfected with HEM45-GFP showed nucleolar staining in addition to predominant cytoplasmic expression. This was not seen in cells transfected with unfused-GFP constructs (not shown). There are some similarities in expression of -HEM45-GFP and HEM45 antigen (eg cocooning of nucleus in right panel Fig. 4 and in prophase Fig. 2b). We did not see nucleolar staining with antisera, where studies have focused on the active cell cycle. We shall now be able to address localization more comprehensively in studies of living cells proposed for section D.3.b.

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The stable cells lines were subjected to flow analysis, and to synchronization by serum starvation (which we find is not fully effective in synchronization of HeLa. Preliminary data suggests that serum stimulation of the starved GFP-HEM45 cell lines increases the mean fluorescence of cells (see Fig. 5) with both the low fluorescence phenotype and the cells with brighter fluorescence. The fraction of cells with the bright phenotype increases in serum stimulated cells. We analyzed for both cell cycle stage and GFP in ethanol fixed cells (see Fig. 6). There is some specific enrichment of the bright GFP fluorescence population for G2/M cells. We find this to be in agreement with our analysis of HEM45 antigen (proposal section C.6), and we propose that the cell regulates the level of HEM45-GFP in a similar manner (possibly independent of the mRNA level in the cell cycle). However, we need to prove that this behavior is specific to the fusion, and is not due to serum stimulation of the GFP-cassette's CMV promoter nor to regulation specific to the GFP moiety. We realize that, for comparison purposes, we need a cell line stabley transfected with the unfused GFP. Note that the HEM45-GFP behaves differently to GFP, suggesting HEM45 affects cell associations of the fusionprotein; cell cycle analysis of unfused GFP is not possible in ethanol fixed cells - the GFP leaches (Paraformaldehyde fixation/triton permeablization offers an alternative methodology for cell cycle flow analysis, this is not a problem for microscopy of living cells).

These studies are continuing, with the intention of evaluating HEM45 in living cells through the cell cycle, and evaluating fusion protein control under additional methods of synchronization, and perhps under action of agents that affect cell cycle progess (DNA damaging agents, and other agents that might invoke a G2 arrest)

The HEM45-GFP fusion lines should be a useful adjunct for cell cycle analysis proposed in section D.2.a.i and studies on the relevance of specific HEM45 motifs to trafficking etc. (eg. see proposal section D.2.a) It is relatively easy to create new cloned lines (of mutant HEM45-GFP) in HeLa.

Plan for the next year: Additional items
D.3. Relationship to proliferation: models for postconfluent breast tumor cell growth
Drs Depasquale and Gierthy of this facility utilize a model of cell post-confluent cell growth (22) that leads to formation of a multi-cellular foci in confluent MCF-7 monolayers. These Foci are induced by estrogen treatment of cultures and there is some evidence that these from by continued cell proliferation. It will be a in-vitro useful model for assessment of HEM45 antigen (and/or HEM45 GFP fusion) expression in a setting (unlike most tissue culture models) where proliferation is occurring but is disassociated from any cell-contact effects of pre-confluent growth. This distinction is relevant and is possibly equivalent to the difference between wound-healing and cell growth in a solid tissue setting. In a preliminary experiment the PI found immunohistochemical staining at a gross level associated with foci. However the enhanced staining of foci could be due to trapping of histochemistry reagents in the multilayer foci, it was clear that better imaging approaches offered by flourescent and confocal microscopy was needed- these resources are available locally as we are home to NIH biomedical imaging regional resource and will be done in collaboration with Dr Depasquale. In parallel, we can also determine if HEM45 mRNA over-expression, using the MCF-7 lines developed as parts of tasks 1-6, affects foci formation, morphology etc.

D.4. Demonstrating biochemical actions of HEM45
Recent advances in the homology data for HEM45 allows an analysis of the function of HEM45 using recombinant protein developed in Task 10 of the IDEA. The Protein developed in task 10 will be used in studies of HEM45 role using biochemical and biophysical approaches. The HEM45 ORF encodes a 20,300 kDa protein of 181 aa residues. The exonuclease features shared with the proof reading domain

of DNA polymerase (see section C.3) encompass the whole of protein barring the first and last half-dozen amino acids, much as seen for the cyclically controlled leukemia cell OP18. This suggests that this feature forms the essence of structures in HEM45. This aim intends to determine if HEM45 is a nuclease and/or if it associates with nucleic acid. We can envisage the motif being used as an exonuclease, or being 'recycled' as a structure with affinity for nucleic acid. We also note that HEM45 should have inherent non-specific affinity for DNA via the high positive charge associated with the C-terminal regions of the protein. Biophysical studies may also be undertaken of HEM45 with other proteins (*e.g.* Tubulin) and itself.

D.4.i **HEM45 expression System**
We do need to improve/optimize HEM45 expression to facilitate studies and to ensure access to adequate pure protein . This involves switching the ORF to a Kanamycin resistant vector, allowing better maintenance in cells, and a more rigorous evaluation of expressing host cells: We used a Lys E variant of E. coli strain BL21, this offers tight control of pET vector expression- but limits protein yield. It would also be more convenient if we could establish conditions that gave soluble protein, rather than inclusion bodies (this is an empirical optimization).

D.4.ii **Biochemistry of HEM45:**
The HEM45 protein will be tested against a variety of radiolabeled nucleic acid substrates using classical tracer solubilization assays (see below). Moser and co-workers (7) noted that one member of the sequences subgroup in which they placed HEM45 had nuclease activity (PAN2 subunit, see section C.3). This activity was against RNA, though they also noted that sequence-based sub-groups were not predictive of the nature of nucleic acid substrates.

Templates: The templates will include linear ds plasmids labeled at 3' ends by fill in of 5' overhangs with α ^{32}P-dNTPs and Klenow and fragments labeled at 5' ends by polynucleotide kinase and γ^{32}P-ATP. Body labeled DNA's will be prepared by generation of a complementary strand on ssDNA of rescued phagemid (*eg* pSelect) or by PCR reactions including a labeled nucleotide. We have previously made labeled single stranded DNA probes for in-situ work (23) using synthetic cold riboprobes as template for reverse transcriptase with radiolabeled nucleotides. This approach could be used to generate DNA-RNA hybrid substrates. All labeled DNA probes will be phenoled and subjected to purification on Sephadex. We shall generate labeled RNA probes with T3/T7 RNA polymerase, though with RNA there is a need for much greater rigor to prevent accidental degradation of substrate at all points in experiments.

There is the question as to selectivity for substrate, and effectiveness with the provided substrates: this simply has to be addressed experimentally and can aid in the elucidation of the cellular role of HEM45. The protein may have activity on multiple substrates. We note that Rec 1 was recently characterized (24) as a 3'-5' exonuclease on ordinary DNA templates, yet previous studies (11) showed a strong affinity to Z-DNA and this was thought significant for action in recombination. In addition the HEM45 relative Sep1 of *S. cerevisiae* was identified as both a 5'-3 'exonuclease and as 5'-3' exoribonuclease (also active on ds-RNA and DNA-RNA hybrids (12)) and thought to have a role in recombination; it was later identified as a DNase active on G4-tetraplex DNA (cutting at adjacent ss regions) by Wally Gilbert (25) - this alternate DNA structure being found associated with G-rich telomeric regions.

Assaying and optimizing putative nuclease activity of HEM45: Parameters to be addressed in assessing HEM45 nuclease activity are summarized in Fig. 7. As a starting point 100 µl assays will include protein extract diluted to not exceed 10 nM NaCl (1 in 10 dilution or greater), buffered with 20 mM Tris HCl, pH'd as appropriate, with 1mM DTT to stabilize thiol groups, and $MgCl_2$, 10mM. Substrate will be added at approximately 1 pmol of ends with a minimum of 50,000 dpm radioactivity Reaction volumes

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will be pre-equilibrated at 37 prior to introduction of protein, and incubated at 37. Reactions will be terminated with 200µg salmon sperm in 200µl cold 10%TCA, mixed, stood on ice 10 min, centrifuged 10,000g for 10min and the soluble material, representing digested substrate, taken for scintillation counting. Routine controls will included protein blanks and samples to which DNase I is added as protein. Results can be converted to pmol DNA(ends)/µg protein/min if required.

An obvious fear is that nuclease activity detected in the assays will actually be from contaminating *E. coli* protein. Eventually we shall demonstrate co-purification of the nuclease activity and HEM45 protein through the process of purification to homogeneity. However, as a rapid comparative control we will include extracts of cells carrying other sequences in pET vectors. We have available a control construct containing β-galactosidase, which should give a soluble protein , and a His tag construct to transcription factor Sp-1 from which we previously isolated protein in inclusion bodies.

D.4.iii. Biophysical properties of HEM45
The Wadsworth Center Biochemistry core offers convenient access to a range of modern biophysical techniques that are applicable to analysis of HEM45. Beyond simple measures of protein structure, biophysical approaches will also offer a means of assessing HEM45 interaction with nucleic acid (NA) substrates without the necessity of the substrate being digested. We envisage initially probing for any interaction with salmon sperm DNA and then assessing relative affinities for a variety of nucleic acid substrates, including DNA and RNA (single-, double- stranded and hybrids as summarized in Table 1). Fluorescence of the tryptophan will provide a sensitive probe of changing conformation, with the possibility of signal enhancement or quenching as the ring becomes more embedded or surface oriented within NA-associated HEM45. Presence of a single tryptophan in HEM45 will greatly aid interpretation. Fluorescence analysis is carried out on a Perkin-Elmer LS50B luminescence spectrophotometer Stoichiometry of protein-NA interactions may be addressed by observation of change in the fluorescence spectrum. Alterations in protein conformation upon association with NA may also be addressable using circular dichroism, but there will be difficulty in interpreting the data if interaction also affects substrate conformation. DLS is again an effective tool in demonstrating interactions, for example between NA and protein.

E. CONCLUSION: A ROLE FOR HEM45 IN CELL DIVISION
The revised focus for our studies is summarized in Fig. 8. Co-localization of HEM45 with mitotic spindle and repeated linkage of HEM45 to proliferative settings suggests a role for HEM45 in cell replication. This is bolstered by HEM45 homology to the protein family that also encompasses the proof reading exonuclease function of Pol I which includes peptides with known roles in replication and action in the mitotic period. The continuing studies should define both the function of HEM45 and its mechanism of action. Unfortunately, the PI is limited in the amount of time he can apply to the project, above the 30% allocated, and with one other funded person progress will be limited. The postdoc carrying out the studies on tet-regulated lines did not return from maternity leave, the PI is currently hiring a technician to continue studies in the approved no cost extension period. However, some proposed studies are collaborative, maximizing everyone's effectiveness and the described studies provide the core of other grant proposals submitted in summer 98.

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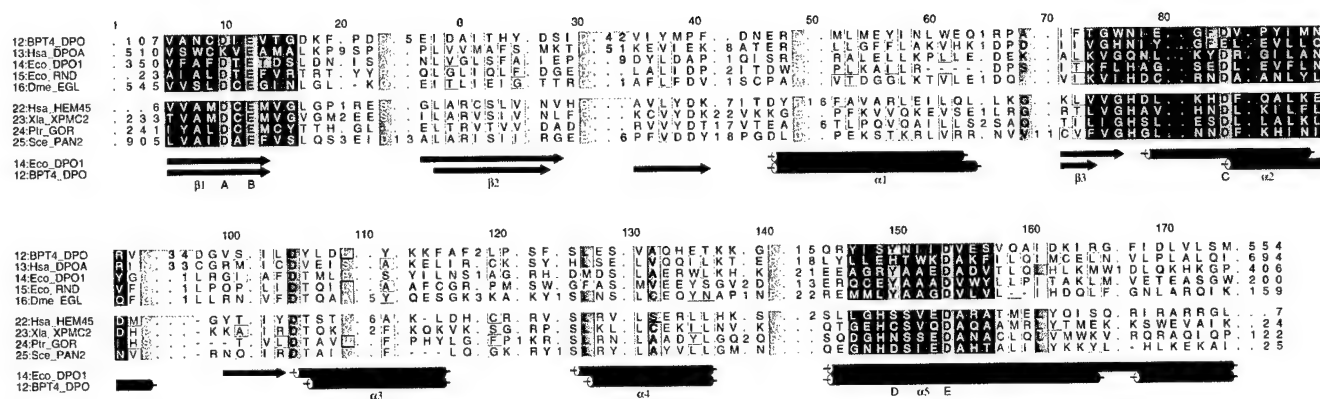
APPENDICES

Fig. 1. HEM45 has sequence motifs relating it to the proofreading 3' 5' exonuclease of E.coli DNA Pol I
Data excerpted from color Fig.1 of Moser et al (Nucleic Acid Res 24:5110-18,1997). The three largest
shaded blocks (residues 4-15, 71-90, 147-158) represent the conserved 'exo domains' recognized in
Polymerase exonucleases. Lighter shaded areas mark major gaps for some seqs. Within the exo motifs are
strongly conserved acidic (Asp/Glu) residues, tagged ABCE under the alignment. These coordinate with
metal ions in the crystalized Pol1 molecule. The active site in the Pol 1 exonuclease is the tyrosine (Y) at 150
(tagged D under seq). This is missing in the HEM45 subfamily; probably replaced by the histidine (H) at the
preceding position.
12. Bacteriophage T4 pol proof-reading subunit. 13. Human DNA pol α proof reading domain, 14. E.coli
Pol1 14 E.coli RnaseD15. Secondary structures of 12 and 14 are shown below the alignment. These are
derived from solved structures.
Sequences 22-25 form a subgroup. This is the HEM45 family that we previously recognized. The yeast
Rnase is an addition, and suggests this group may have nuclease activity.
22. HEM45. 23. XPMC2: Xenopus seq, rescues cell cycle control defect in S. pombe model. 24. CHPGOR
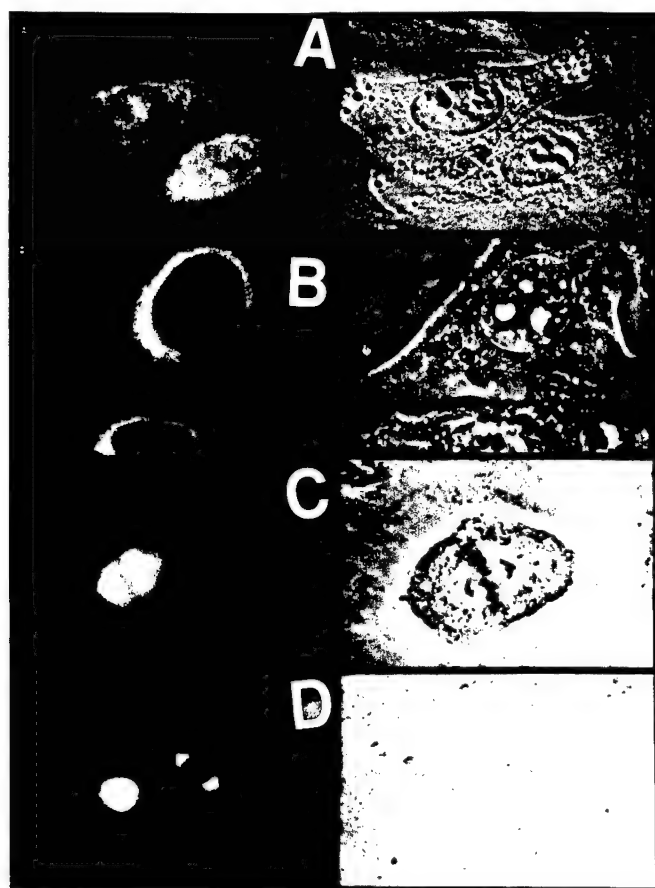gorilla nuclear auto-antigen. 25. PAN2 *S cerevisiae* polyA shortening RNase subunit.

Fig. 2. Localization of HEM45 in synchronized HeLa.
Cells at specific cycle stages were stained with Y29 anti-HEM45 Ab and FITC 2ndary: (A) Interphase; (B) Prophase; (C)Metaphase; (D) Anaphase; Right panel: phase contrast images. 200x mag. A cell population enriched for mitotic cells was generated by double thymidine block and release.
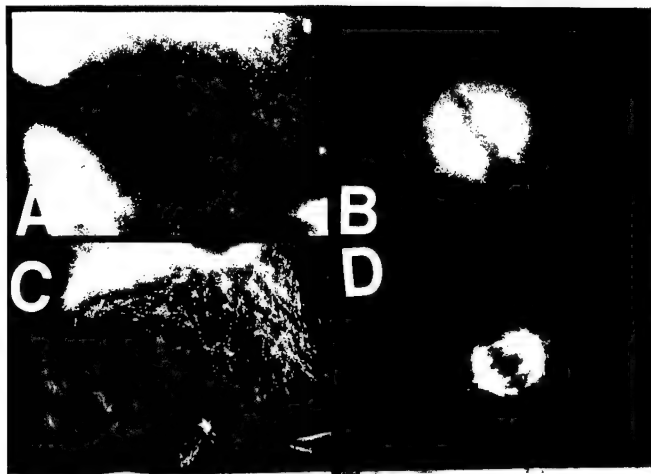
Fig. 3. Comparative HEM45& tubulin localization in HeLa.
Cells were stained with rabbit anti-HEM45 (A,B) and mouse anti-tubulin (C ,D) antibodies. Interphase cell A&C. Metaphase cell:B&D. Cells were stained with individual primary antibody (1:500) for 1 hour at room temperature , followed the appropriate FITC secondary. Cells were then restained with the second primary-2ndary. Photographed at 100x. HEM45 interphase staining is distinct from microtubules. Mitotic HEM45 overlays spindle structures, but has distinct staining pattern
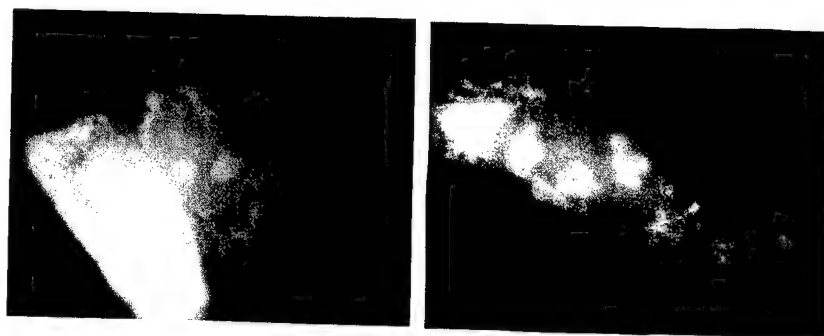
Fig. 4. HEM45-GFP expression in a cloned puromycin resistant line carrying a fusion construct: Two positive images from fluorescence Microscopy.

HEM45 ORF was placed N-terminal to GFP in pGEFP-N1 (Clontech) and stable lines derived by pUR co-transfection and puromycin selection (as Pentecost, J steroid Biochem, 64, 25-33, 1998). HEM45-GFP expression was evaluated by Dr DePasquale in live cells using a Zeiss Axiovert 135 microscope equipped with a 100X (1.3 NA) Plan-Neofluar objective lens, fluorescein filter set (450-490ex/515-565em), and a 100 W mercury arc illuminator. (Note: in some cases cells were also evaluated with a phase objective under the same illumination conditions; we detected no deleterious effects on cells as judged by overall cell morphology). Cells were maintained at 37° at the stage with an air-curtain incubator. Images of HEM45-GFP fluorescence were acquired by on-chip frame integration using a Dage CCD-300-RC camera operated by a Scion VG5 frame-grabber board and ScionImage PC software running on a Dell P350 computer.
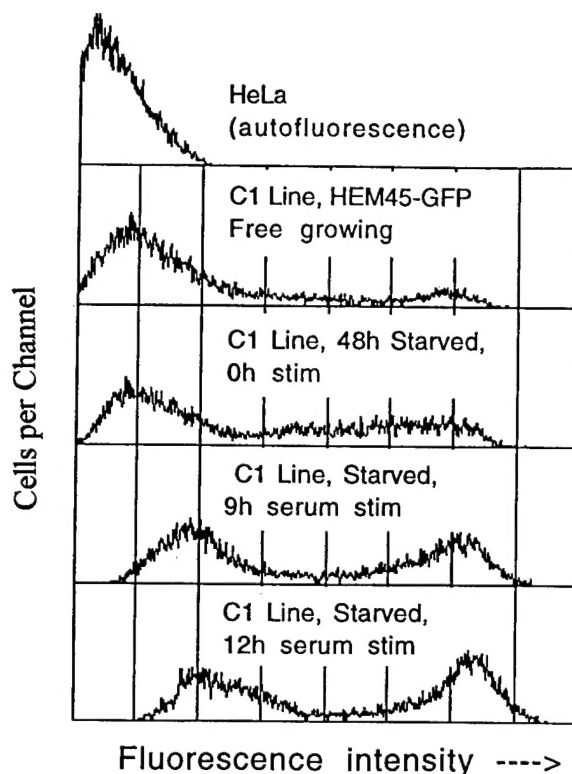
Fig. 5. HEM45-GFP expression in stable transfect GST-C1 derived from HeLa: Flow cytometry.
The GST- C1 line has higher low level fluorescence than the parent HeLa line, a subset of GST-C1 cells glow brightly. The general fluorescence increases following release to cell cycle upon serum treatment. The bright fraction especially increases. The bright fraction in serum stimulated cells appears moderately enriched for G2/M (See sup fig). This continues the concept of HEM45 having a role in the cell cycle/cell proliferation.
GFP fluorescence was determined in cells formaldehyde fixed cells by flow cytometry, cell culture treatments are as described for cell cycle analysis. HeLa cells are the unsynchronized parent line to GST-C1, and give a measure of autofluorescence. The X-axis gives a measure of fluorescence intensity, the Y axis indicates the fraction of cells with a particular intensity.

Weakly fluorescing cells	G0/1	S	G2/M
free cycling	57%	17%	26%
blocked, 0h stimulated	55%	17%	28%
blocked, 9h stimulated	52%	24%	25%
Bright fluorescing cells			
free cycling	60%	15%	25%
blocked, 0h stimulated	49%	16%	**35%**
blocked, 9h stimulated	41%	24%	**35%**

Fig. 6. Cell cycle analysis of Ethanol fixed HEM45-GFP fusion cell line GST-C1.
Cells of indicated treatment were ethanol fixed and then processed together for cell cycle analysis using propidium iodide staining. '2-color' analysis was used to simultaneously evaluate cell cycle status and GFP fluorescence. Note that HEM45-GFP remains associated with ethanol fixed cells- in contrast to generally reported behavior of unfused GFP. These cells were from the same cell cycle synchronization experiment as shown in sup fig. 3, the 12 h time point was lost but we know from experience that the 9 h stimulation period gives maximal enrichment for S phase, these cycle through to give a maximal G2/M at 12 h. HeLa do not fully synchronize in response to serum starvation (Knehr etal Exp Cell Res 217546-53, 1995), but it is the least perturbing method for mass synchronization. For synchronization, HeLa derivative cells were plated at 250K/T25, grown for 18 h in 10% Serum, switched to 0.3% serum for 48 h and then returned to 10% serum for the indicated period.

Fig.7: Parameters for HEM45 as a Nuclease
Substrate: DNA ss,ds, end and body-labeled, RNA, hybrids
pH: 5-10,
Time: linearity? Temp 23-42°
Protein extract: linearity with input
Ions: Mg, Ca, Na, K,
Salt(NaCl) 0-100mM
(nucleotide cofactors/competitors ATP, dAMP dATP.....)
(Chelating inhibitors: EDTA, Phenanthrolene)
For comparison: work up βGal and Sp-1 expression
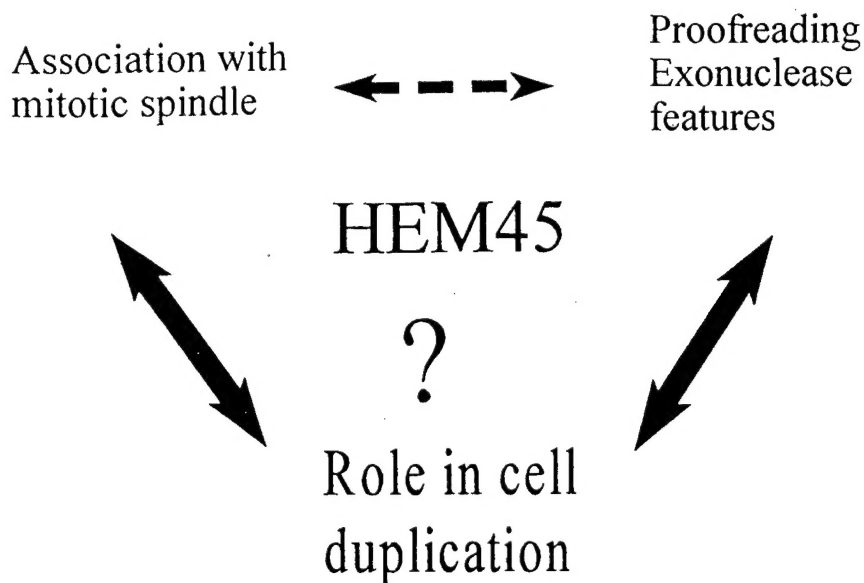constructs in parallel (control for *E. coli* nuclease)

Association with
mitotic spindle

Proofreading
Exonuclease
features

HEM45

?

Role in cell
duplication

Fig. 8. Integrative model for studies of HEM45.
We propose that HEM45's function is in the process of cell division, with a specific metaphase role.
Moreover we hypothesize that this function involves the potential exonuclease motif identified in HEM45
by sequence comparisons. We know HEM45 associates with mitotic spindle structure, again we can posit
that action is related to this association. We predict variable expression and phosphorylation of HEM45
in the cell cycle.